



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : G01N 33/53, 33/577, 33/82 C07K 15/28	A1	(11) International Publication Number: WO 91/00519 (43) International Publication Date: 10 January 1991 (10.01.91)
(21) International Application Number: PCT/US90/03416 (22) International Filing Date: 15 June 1990 (15.06.90) (30) Priority data: 371,234 26 June 1989 (26.06.89) US (71) Applicant: TRITON BIOSCIENCES, INC. [US/US]; 1501 Harbor Bay Parkway, Alameda, CA 94501 (US). (72) Inventors: POURFARZANEH, Mohammed, T. ; 37 Shannon Circle, Alameda, CA 94501 (US). KATSILOMETES, George, W. ; 16 Durango Court, Sacramento, CA 95833 (US). (74) Agent: SCHUURMAN, Willem, G.; Arnold, White & Durkce, P.O. Box 4433, Houston, TX 77210 (US).	(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published - With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(54) Title: VITAMIN B ₁₂ ASSAY (57) Abstract Immunoassays for vitamin B ₁₂ using novel monoclonal antibodies to the intrinsic factor: vitamin B ₁₂ complex and to the vitamin B ₁₂ binding site on intrinsic factor.		

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VITAMIN B₁₂ ASSAY

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Vitamin B₁₂ (cyanocobalamin) has an empirical formula of C₆₃H₈₈O₁₄N₁₄PCo (Mol. Wt. 1355.4 daltons) and is a complex molecule having three major components: a porphyrin-like ring structure consisting of four reduced and extensively substituted pyrrole rings (designated A to D) surrounding a single cobalt atom; an α-5,6--dimethylbenzimidazole ribonucleotide group which links to the corrin nucleus with bonds to the cobalt atom and to the propionate side chain of the D ring; and a variable R group which can be cyanide, hydroxyl, methyl or 5-deoxyadenosyl. The cyano-substitution is an artifact of isolation but is the form which is currently assayed. Hydroxycobalamin, methylcobalamin and 5-deoxyadenosylcobalamin are the physiologically active coenzyme forms of vitamin B₁₂. The two major active cobalamins, methylcobalamin and 5-deoxyadenosylcobalamin, are essential for DNA biosynthesis, replication and cell growth.

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Dietary vitamin B₁₂, in the presence of gastric acids and pancreatic proteases, is released from intestinal R protein-like binding proteins and is immediately bound to intrinsic factor (IF), the gastrointestinal binding glycoprotein responsible for the specific binding and absorption of vitamin B₁₂. Intrinsic factor has a molecular weight of approximately 40,000 and differs markedly in structure from the R proteins. Its ability

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to bind vitamin B₁₂ at the exclusion of metabolically inactive cobinamide has led to the predominant use of this binding protein in the commercially available vitamin B₁₂ assays. The IF:vitamin B₁₂ complex interacts with the ileal mucosal cells and is then absorbed into the blood stream.

Following absorption, all three active cobalamins are present in plasma bound to plasma-binding proteins known as transcobalamin-II and R proteins (also known as nonintrinsic factors). Transcobalamin II binds approximately 20% of the total plasma vitamin B₁₂ while the remainder is bound to the R proteins. Vitamin B₁₂ bound to transcobalamin II, is rapidly cleared from the plasma and delivered to a variety of cells, such as the hepatic parenchymal cells of the liver. The R proteins may also play a role in the delayed transport and cellular delivery of vitamin B₁₂ to the liver. Because of the high affinity with which the plasma-binding proteins bind vitamin B₁₂, the denaturation of these proteins is necessary in the performance of cyanocobalamin assays.

Vitamin B₁₂ deficiency is due primarily to a disruption of IF:vitamin B₁₂ binding resulting from a failure of parietal cell production of intrinsic factor or the production of anti-intrinsic factor antibodies. The failure of parietal cells is probably due to the presence of cytotoxic autoantibodies. Serum antibodies to intrinsic factor have been extensively studied. Two types of antibodies have been identified: the blocking antibody (Type I), which blocks the binding of vitamin B₁₂ to IF and a nonblocking antibody (Type II), which reacts with the IF:vitamin B₁₂ complex. Serum blocking antibodies are present in 50-76% of vitamin B₁₂ deficient patients and have a high degree of specificity for the diagnosis of pernicious anemia. Commercial assay kits which measure the levels of these anti-IF antibodies are

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available (Corning ImmoPhase IFAB) and studies on the utility of these assays in the differential diagnosis of vitamin B₁₂ deficiency have proceeded to the present.

5 Vitamin B₁₂ deficiency is more commonly caused by defective gastrointestinal absorption than from a deficient diet. Therefore, additional factors which can lead to vitamin B₁₂ malabsorption include the combination of gastric achlorhydria and decreased secretion of IF
10 secondary to gastric atrophy or surgery, intestinal parasites, bacterial gastroenteritis and intestinal malignancies. Other disorders which may account for a vitamin B₁₂ deficiency include dietary deprivation (veganism), drugs, organ disease (liver), hyperthyroidism
15 and transcobalamin II aberrations.

A deficiency in vitamin B₁₂ can lead to disease states marked by defective DNA synthesis culminating in
f delayed cellular mitosis. Since the cells having the most rapid metabolism will be the most dramatically
20 affected, the hematopoietic system is particularly sensitive to vitamin B₁₂ deficiencies. The primary clinical manifestation of this vitamin deficiency is the development of a megaloblastic anemia which is also known
15 as pernicious anemia. Vitamin B₁₂ is also implicated in the maintenance of the myelin of the nervous system. For these reasons, it is very important to be able to diagnose vitamin B₁₂ deficiencies.

10 The current state of the art regarding vitamin B₁₂ assays utilizes a wide variety of assay techniques. Microbial assays are based upon the principle that some microorganisms such as Euglena gracilis and Lactobacillus leichmannii require vitamin B₁₂ for normal growth and
15 replication. Standard curves are produced using known amounts of vitamin B₁₂ and bacterial growth produced by a sample containing an unknown amount of vitamin B₁₂ is

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compared with the standard curve. Bacterial growth can be measured in numerous ways but the most common is turbidimetric measurement.

5 The deoxyuridine suppression test is performed on bone marrow cells which have been aspirated from the patient. A vitamin B₁₂ deficiency is noted if there is a reduction in the suppression of radioactive iododeoxyuridine incorporation into DNA by deoxyuridine.

10 High-performance liquid chromatography is based upon the variability in the rate of travel of different compounds through a medium under pressure within a column. The elutes are then detected at picogram/ml concentrations by ultra-violet or other detectors such as

15 fluorescence and electrochemical detection.

 Another method involves the use of antisera specific to vitamin B₁₂ which are employed in specific

20 radioimmunoassays to directly measure the liberated vitamin.

 Still another method involves competitive protein-binding radioimmunoassays (radioisotopic dilution

25 assays). These assays have the prerequisite that the vitamin B₁₂ in the sample be freed from its binding proteins by physical, chemical or enzymatic denaturation or digestion. The most commonly used methods are the

30 boil method which involves boiling the sample for 15-30 minutes and the non-boil method which involves the use of highly alkaline buffer solutions containing KCN which simultaneously denature the binding proteins and convert the cobalamins to the stable cyanocobalamin form. Both

35 the boil and the non-boil methods use radioactive (⁵⁷Co-labelled) vitamin B₁₂ in a competitive system which employs purified hog intrinsic factor as the binding

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agent and a separation step to remove unbound sample vitamin B₁₂.

5 In spite of the fact that there are numerous vitamin B₁₂ assays currently available, there remains a continuing need for an improved assay that will overcome the shortcomings of the state of the art techniques. These shortcomings include a lack of sensitivity, difficulties encountered in standardization and the impracticality of use in high volume situations, i.e. in hospitals. 10 Further, there are no commercial non-isotopic immunoassays for this analyte on the market. During the past decade, major attempts have been made by numerous groups to produce such a non-isotopic test for vitamin B₁₂. 15 These attempts have been unsuccessful, due primarily to the small size and complexity of the vitamin B₁₂ molecule. Therefore, there is a current need for an accurate, precise and reliable reference method for assaying cyanocobalamin. This invention is intended to address that need. 20

This invention pertains to the development of a process and an immunoassay for the determination of the concentration of vitamin B₁₂ in a sample. The process can 25 be performed manually such as by means of a diagnostic kit or automatically such as in an automatic analyzer.

10 In particular, this invention in one aspect pertains to the production and isolation of monoclonal antibodies specific to the intrinsic factor: vitamin B₁₂ complex and in another aspect to the production and isolation of monoclonal antibodies specific to the vitamin B₁₂ binding site on intrinsic factor. Further, this invention is directed at the production and isolation of monoclonal 15 antibody lines capable of producing these antibodies.

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Figure 1 is a titer curve for several monoclonal antibody cell lines, depicting the percentage of ^{125}I -IF bound versus the amount of antibody dilution.

5 Figure 2 is a bar graph illustrating the effect of different monoclonal antibodies on vitamin B_{12} binding to intrinsic factor.

10 Figure 3 is a calibration curve illustrating the specificity of the monoclonal antibodies of this invention for the vitamin B_{12} binding site on IF.

15 This invention provides for the development of a process and an immunoassay for determination of the concentration of vitamin B_{12} in serum or other body fluids which involves the use of novel monoclonal antibodies. The preferred embodiment of this invention pertains to the production of monoclonal antibodies specific to the vitamin B_{12} binding site on intrinsic factor. In a competitive assay, vitamin B_{12} would compete with labelled monoclonal antibodies for binding to intrinsic factor. Use of these site-specific antibodies in such an assay, enables indirect measurement of vitamin B_{12} , as the vitamin B^{12} levels are measured by monitoring antibody binding to IF. The amount of antibody bound is inversely proportional to the amount of vitamin B_{12} present. The sample is first boiled or pretreated with a denaturing solution (non-boil method) to release vitamin B^{12} from its binding proteins, to which it is strongly bound.

25 Following denaturation, the "site specific" monoclonal antibody reagent and labelled intrinsic factor are added and incubated with the sample.

35 Another embodiment of the invention pertains to the production of monoclonal antibodies specific to the IF: vitamin B_{12} complex and use of these antibodies to identify the IF: vitamin B_{12} complex. Sample preparation

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is identical to that for the embodiment described above. Binding of the sample vitamin B₁₂ is accomplished by labelled IF. The antibodies to the IF:vitamin B₁₂ complex are labelled for use in identification of the IF:vitamin B₁₂ complex and separation is accomplished by standard methods. Monoclonal antibody binding is in direct proportion to the amount of sample vitamin B₁₂ present.

Labelling of intrinsic factor and the monoclonal antibodies can be done by incorporating labels commonly used in state of the art immunoassays, such as hapten molecules, radioactive atoms or groups, enzymes, vitamins (biotin), dyestuff or fluorescent groups. This labelling is usually performed before any immunological reaction is allowed to take place. However, it may be performed at a later stage by allowing the component which must be labelled to react with the corresponding binding partner, which has previously been labelled. The coupling of the enzyme/dyestuff/fluorogen can be brought about in a known way, for example by the formation of an amide linkage. Labelling with a radioisotope can also be performed by methods well established in the art. Useful isotopes include, without limitation, ¹²⁵I, ¹³¹I, ¹⁴C and ³H.

In the preferred embodiment, one of the labels used is a signal generating label for use in detection and the other label is capable of being captured by a capture ligand on a solid phase. For example, intrinsic factor can be labelled with an enzyme and the monoclonal antibodies labelled with a hapten, or vice versa. Preferably, the enzymes are those which can be colorimetrically, electrochemically, spectrophotometrically or fluorometrically determined. These include, without limitation, alkaline phosphatase, acetylcholinesterase, malate dehydrogenase, egg white lysozyme, horseradish peroxidase and β -galactosidase. The preferred hapten

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molecule is a fluorogenic molecule, for example fluorescein isothiocyanate (FITC).

5 The preferred solid phase is magnetizable particles, but this invention also contemplates the use of filter paper, plastic beads, or test tubes made from polyethylene, polystyrene, polypropylene or other suitable materials. Also useful are particulate materials such as agarose, crosslinked dextran, and other polysaccharides. Techniques for binding to the solid phase are also well known in the art. Numerous solid support materials and binding methods can be used, examples of which are described in U.S. Patent No. 4,659,678, incorporated herein by reference.

15 Separation of the complexes may be by any of a variety of physical, chemical or immunological techniques. Likewise, any of the common substrates known in the immunoassay art may be used. The preferred separation procedure uses antibodies linked to magnetizable particles. Examples include the fluorescein antibody magnetizable particle (FAMP) method as described in S.J. Rattle et al., Clin. Chem. 30(9):1457-1461 (1984) or double antibody magnetic particle separation, using anti-mouse labelled magnetic particles as described in U.S. Patent No. 4,659,678. Another suitable separation procedure is the covalently linked antibody magnetic particle separation method, which involves mixing the antigen-containing sample with two monoclonal antibodies raised against different epitopes of the antigen, where one monoclonal antibody is enzyme labelled and the other is covalently bound to a polyclonal antibody raised against FITC, which is subsequently covalently bound to magnetizable particles. Other separation procedures may also be suitable.

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In both embodiments of the present invention, it may be necessary to utilize labelled antibodies specific for alternate determinant sites on IF in order to identify the (IF:vitamin B₁₂) (monoclonal antibody) complex.

It is possible to amplify the sensitivity of immunoassays using monoclonal antibodies of this invention in numerous fashions. Typical examples are enzyme amplification, Stanley et al., Am Biotechnol. Lab. 3:48-53 (1985), and the biotin-avidin method, L.A. Sternberger, Immunocytochemistry, 2nd ed., John Wiley & Sons (1979). These designs will theoretically yield the desired sensitivity because the primary reagents are combined in solution prior to separation.

The vitamin B₁₂ immunoassay utilizing monoclonal antibodies to the vitamin B₁₂ binding site on IF involves the following steps:

treating a sample of the fluid to free vitamin B₁₂ from the binding proteins;

contacting the sample with labelled monoclonal antibodies specific to the vitamin B₁₂ binding site on intrinsic factor and labelled intrinsic factor, to form intrinsic factor:antibody and intrinsic factor:vitamin B₁₂ complexes;

adding a solid phase to said complexes to form bound and unbound complexes;

separating the bound and unbound complexes and washing; and

measuring either the amount of bound complex or unbound complex to quantify the amount of vitamin B₁₂ present in the sample. If the amount of

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intrinsic factor:antibody is measured, the value will be inversely proportional to the amount of vitamin B₁₂ present in the sample. If the intrinsic factor:vitamin B₁₂ is measured, the value is directly proportional.

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Accordingly, an immunoassay of this embodiment of the invention would comprise (1) monoclonal antibodies that bind to the vitamin B₁₂ binding site on intrinsic factor and block the binding of vitamin B₁₂ to intrinsic factor said monoclonal antibodies being labelled with a first label, (2) intrinsic factor labelled with a second label and (3) a solid phase containing a capture ligand for one of said labels with the other label being a signal generating label for use in detection. The process itself is illustrated in Example 1. For purposes of illustration only, the monoclonal antibodies are labelled with FITC, intrinsic factor is enzyme labelled. Separation is done by the FAMP method, so magnetizable particles are used as the solid phase with antibodies specific to FITC being the capture ligand. These steps are not intended to limit the invention in any manner but are merely illustrative of an assay that can be developed using these novel monoclonal antibodies.

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EXAMPLE 1

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1. The sample containing the vitamin B₁₂ (B₁₂) analyte bound to the binding proteins, is chemically or physically (e.g. heat or change in pH) pretreated. This pretreatment frees the analyte, resulting in a sample containing free vitamin B₁₂ and the denatured binding proteins.

2. The sample is mixed with FITC conjugated monoclonal antibodies (FITC-MAb) which are specific to the IF binding site and subsequently incubated with enzyme labelled

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intrinsic factor (IF-EZ). This is a competitive step since the vitamin B₁₂ "competes" with the monoclonal antibodies for binding to IF.

5 B₁₂ + FITC-MAb + IF-EZ EZ-IF:MAb-FITC + EZ-IF-B₁₂

3. Antibodies specific to the label used on the monoclonal antibodies; in this instance FITC (α-FITC), coupled to magnetizable particles (MP) are added in excess.

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MP-α-FITC + EZ-IF:MAb-FITC MP-α-FITC:FITC-MAb:IF-EZ

4. The captured complexes are then separated by magnetic particle separation and washed to remove the free IF-EZ.

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5. The signal from the MP-bound complex is then measured spectrophotometrically following the addition of a substrate reagent such as pNPP and/or an amplification reagent. The color intensity obtained is inversely proportional to the vitamin B₁₂ concentration.

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15 The vitamin B₁₂ immunoassay utilizing monoclonal antibodies to the IF:vitamin B₁₂ complex involves the following steps:

treating a sample of the fluid to free vitamin B₁₂ from the binding proteins;

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contacting the sample with labelled intrinsic factor, in excess, and labelled monoclonal antibodies specific to the intrinsic factor:vitamin B₁₂ complex, in excess, to form intrinsic factor:vitamin B₁₂:antibody complexes;

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adding a solid phase to said complexes to
form bound and unbound complexes;

5 separating the bound and unbound complexes
and washing; and

measuring either the amount of bound
complex or unbound complex to quantify the amount of
vitamin B₁₂ present in the sample. If the amount of
10 intrinsic factor:vitamin B₁₂:monoclonal antibody is
measured, the value will be directly proportional to the
amount of vitamin B₁₂ in the sample. If the amount of
excess monoclonal antibodies is measured, the value will
be inversely proportional.

15 Accordingly, an immunoassay of this embodiment of
the invention would comprise (1) monoclonal antibodies
that bind to the intrinsic factor:vitamin B₁₂ complex said
monoclonal antibodies being labelled with a first label,
20 (2) intrinsic factor labelled with a second label and (3)
a solid phase containing a capture ligand for one of said
labels with the other label being a signal generating
label for use in detection. The process itself is
illustrated in Example 2. The intrinsic factor is
25 labelled with FITC and the monoclonal antibodies are
enzyme labelled. Separation is by the FAMP method. Once
again, these parameters are merely illustrative and are
not intended to limit the invention in any manner.

30 EXAMPLE 2

1. The sample, containing the vitamin B₁₂ (B₁₂)
analyte bound to the binding proteins, is
chemically or physically (e.g. heat or change
35 in pH) pretreated. This pretreatment frees the
analyte, resulting in a sample containing
vitamin B₁₂ and free denatured binding proteins.

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2. The sample is then mixed and subsequently incubated with an excess of FITC conjugated intrinsic factor (FITC-IF) together with an excess of enzyme labelled monoclonal antibodies (EZ-MAb) which recognize the IF: vitamin B₁₂ complex.

10 B₁₂ + FITC-IF + EZ-MAb EZ-MAb
FITC-IF: B₁₂ + FITC-IF + EZ-MAb

3. Antibodies specific to the label used on intrinsic factor, in this instance FITC (α-FITC), coupled to magnetizable particles (MP) are added to the sample in excess to separate the complexes.

20 MP-α-FITC + FITC-IF: B₁₂ + FITC-IF + EZ-MAb

MP-α-FITC: FITC-IF: B₁₂ + MP-α-FITC: FITC-IF + EZ-MAb

4. The captured complexes are then separated by magnetic particle separation and washed to remove the free EZ-MAb.

5. The signal from the labelled monoclonal antibodies in the bound complex is then measured spectrophotometrically following the addition of a substrate reagent such as pNPP and/or an amplification reagent. The color intensity obtained is directly proportional to the vitamin B₁₂ concentration.

The method of obtaining the monoclonal antibodies of this invention is that discussed by Kohler and Milstein in Nature 256:495-497 (1975). Summarized briefly, this process involves injecting an animal with an immunogen

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(Example 3 and 4). The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing in vitro. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen (Example 5). In this manner, the individual antibody species obtained, are the product of a single B cell from the immune animal generated in response to a specific site recognized on the immunogenic substance. This method is best described with reference to the following examples, which are merely illustrative of the present invention and should not be considered as limiting the scope of the invention in any manner.

Preparation of the IF:vitamin B₁₂ complex is accomplished by mixing commercially available highly purified hog intrinsic factor with an excess of vitamin B₁₂, to insure maximum complex formation. The free vitamin B₁₂ is then separated by standard gel filtration techniques or dialysis and the IF:vitamin B₁₂ complex can then be used as the immunogen to produce monoclonal antibodies to the complex.

EXAMPLE 3

The IF:vitamin B₁₂ complex immunogen was prepared using a calculated 1:1 molar ratio of IF to vitamin B₁₂ (1 mg IF binds 30 µg vitamin B₁₂). Eight hundred eighty micrograms of IF (16 nM) was combined with 26.4 µg of vitamin B₁₂ (19.48 nM) in 0.1 M NaH₂PO₄ (pH 7.5). The calculated molar ratio of vitamin B₁₂ to IF was 1.22:1. This combination was brought to 2 ml with monobasic phosphate buffer, covered and refrigerated overnight. The immunogen was then purified by FPLC (Pharmacia). Subsequent UV spectrophotometry on the purified complex

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gave a calculated final molar ratio of 1.2:1 with a 96% recover (850 μ g). The purity of the immunogen was also determined using native PAGE electrophoresis and the molecular weight was determined by comparison with protein calibrators using high pressure liquid chromatography (Hewlett Packard 1090M).

Standard techniques can be employed to select clonal hybridoma cell lines producing monoclonal antibodies to the IF:vitamin B₁₂ complex and to the vitamin B₁₂ binding site on intrinsic factor.

EXAMPLE 4

Eight mice to be immunized with the IF:vitamin B₁₂ complex were divided into two groups of four each. The first group received 100 μ l of immunogen containing 50 μ l of the purified IF:vitamin B₁₂ complex (27 μ g) and 50 μ l of Ribi adjuvant (Ribi ImmunoChem Research, Inc., Hamilton, MT). These injections were given by foot pad and by multiple subcutaneous injections in the inguinal and axillary regions. The second group of four mice received 100 μ l of the same preparation via multiple intramuscular and intraperitoneal routes.

Six mice were selected and divided into two groups of three each for immunization with purified IF. The first group received 100 μ l of immunogen containing 50 μ l (25 μ g) of IF and 50 μ l of Ribi adjuvant/mouse/immunization. These injections were given in the foot pad and subcutaneously at multiple sites in the inguinal and axillary regions. The second group of three received injections of IF at multiple intramuscular and intraperitoneal sites (100 μ l total volume).

A schedule of immunizations and bleeds was established to insure a minimum of 3 bouts of exposure.

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Prebleeds and test bleeds were evaluated for presence of polyclonal antibodies by screening on antigen coated plates (Costar vinyl).

5 Following the third immunization, the mouse having
the highest serum binding to IF (mouse B2) and the mouse
with the highest binding to solid phase IF from the group
receiving the IF: vitamin B₁₂ complexes (mouse D4) were
selected for IV boost and sacrifice. Fusions were
10 carried out using the principles outlines in Kohler and
Milstein, Nature 256:495-497 (1975).

Screening techniques were devised to select specific
hybridoma cell lines capable of producing monoclonal
15 antibodies to the IF: vitamin B₁₂ complex and to the
vitamin B₁₂ binding site on intrinsic factor.

EXAMPLE 5

20 Sera and supernate screening was performed on
duplicate microtiter plates which had been precoated with
100 ng/50 µl well of IF in carbonate-bicarbonate buffer
(50 mM, pH 9.6) and 100 ng/50 µl well of IF: vitamin B₁₂
complex. The plates were incubated at room temperature
25 for 24-48 hours and were then washed three times and
blocked with 52 µl of 2% powdered milk in water. The
plates were then incubated for 1 hour at room temperature
and were rewashed. Fifty microliters of cell culture
supernate from each cell line was added to all the wells
30 and was incubated for 1 hour. The plates were washed and
50 µl of a 1:10,000 dilution of goat anti-mouse-
horseradish peroxidase (HRP) conjugate was added and
incubated for 1 hour. A substrate for HRP (3,3', 5,5'-
tetramethylbenzidine) was then added and the colored
35 reaction product was allowed to form for 30 minutes. The
enzyme-substrate reaction was then stopped by the
addition of 50 µl of 2 M H₂SO₄ and the plates were read at

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450 nm. This screening procedure permitted selection of several lines which had some degree of specificity for either IF or the IF:vitamin B₁₂ complex. This procedure, taken with example 8 permitted selection of lines having some degree of specificity for the vitamin B₁₂ binding site on IF. These cell lines were: 8A4; 6B8; 5G1; 7E4; 2G7; 5E1; 5E11; 1A5; 7H8 and 1G5.

EXAMPLE 6

Intrinsic factor was labelled with ¹²⁵I-Na using the well established chloramine-T method, as set forth in Hunter and Greenwood, Nature (London) 194:495-496 (1962). Intrinsic factor (50 µg in 100 µl distilled water) was mixed with 1 mCi of ¹²⁵I-Na (10 µl, Amersham). Chloramine-T (25 µg/25 µl of 0.1 M NaH₂PO₄ buffer, pH 7.5) was then added and this mixture was incubated for 45 seconds. The reaction was then terminated by the addition of 60 µg (per 50 µl of the phosphate buffer) of sodium metabisulfite. One hundred microliters of the phosphate buffer was then added and the mixture was applied on a PD-10 column (Pharmacia) which had been previously equilibrated with phosphate buffer. The mixture was eluted with the buffer and 0.5 ml fractions were collected in 25 x 75 mm borosilicate tubes. Determination of ¹²⁵I activity peaks indicated that fractions 2 and 3 contained 76% of the activity (38 µg/ml).

EXAMPLE 7

One hundred microliters of cell culture supernate diluted neat, 1:10, 1:100, 1:1000 and 1:5000 were added to duplicate tubes. This was followed immediately by the addition of 100 µl of ¹²⁵I-IF tracer (543 pg/tube) which gave the total tube counts of approximately 10,000 cpm. Following a one hour incubation at room temperature, the

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bound fraction was separated using sheep anti-mouse magnetic particle (2 mg/100 μ l) separation. The bound fractions were then counted on a multichannel radioisotope counter (IsoData 20/20). Figure 1 illustrates the titer curves which were obtained employing the supernates from the cell lines selected (1A5, 6B8, 5E11, 8A4, 7E4 and 5G1).

EXAMPLE 8

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In order to select antibodies specific for the IF:vitamin B₁₂ binding site and/or the IF:vitamin B₁₂ complex, selected supernates were utilized in differential binding studies. Inhibition of ⁵⁷Co-labelled vitamin B₁₂ to intrinsic factor was demonstrated when monoclonal antibody 8A4G11 (expanded clone of the 8A4 cell line) was added in solution with ¹²⁵I-IF. This is shown in Figure 2, which also shows how this monoclonal antibody inhibited binding to a significantly greater degree than other monoclonal antibodies. Another study involved the addition of 100 μ l of 0.1, 1, 10 and 100 ng/ml concentrations of vitamin B₁₂ (in distilled water); 100 μ l of the appropriate dilution of monoclonal antibody supernate and 100 μ l of ¹²⁵I-IF (543 pg/tube) to duplicate tubes, followed by incubation for one hour. The bound complex was separated by addition of anti-mouse magnetic particles (2 mg/100 μ l) after a 15 minute incubation. The calibration curves obtained showed the degree of specificity of monoclonal antibodies for the IF:vitamin B₁₂ complex. Figure 3 shows the calibration curve for monoclonal antibody 8A4G11 further demonstrating the specificity of this monoclonal antibody for the vitamin B₁₂ binding the site on IF. The cell line 7H8 also provided monoclonal antibodies specific to the binding site. One other cell line (6B8) also showed displacement of vitamin B₁₂ which was not as pronounced as that shown by 8A4 and 7H8. The remaining antibodies were either

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specific for IF alone (5G1) or were preferentially specific for the IF: vitamin B₁₂ complex (7E4). The plate and tube screening results led to the initial selection of the following lines for ascites production in pristane primed mice: 8A4, 7H8, 1A5, 6B8, 7E4 and 5G1.

5

CLAIMS:

1. An immunoassay for determining the concentration of vitamin B₁₂ in a sample comprising monoclonal antibodies that bind to the vitamin B₁₂ binding site on intrinsic factor and block the binding of vitamin B₁₂ to intrinsic factor said monoclonal antibodies being labelled with a first label, intrinsic factor labelled with a second label, and a solid phase containing a capture ligand for one of said labels with the other label being a signal generating label for use in detection.
2. Monoclonal antibodies specific for the vitamin B₁₂ binding site on intrinsic factor and having the ability to block the binding of vitamin B₁₂ to intrinsic factor.
3. The monoclonal antibodies of claim 2 which are produced by the cell lines 8A4 and 7H8.
4. A complex wherein intrinsic factor having a first label is bound to a monoclonal antibody specific for the vitamin B₁₂ binding site on intrinsic factor said monoclonal antibody having a second label, where one of said labels is capable of being captured by a capture ligand on a solid phase and the other label is a signal generating label for use in detection.
5. A process for the determination of the concentration of vitamin B₁₂ in a fluid containing vitamin B₁₂ bound to binding proteins, comprising the steps of:
 - treating a sample of the fluid to free vitamin B₁₂ from the binding proteins;
 - contacting the sample with labelled monoclonal antibodies specific to the vitamin B₁₂ binding site on intrinsic factor and labelled intrinsic

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factor, to form intrinsic factor:antibody and
intrinsic factor:vitamin B₁₂ complexes;

5 adding a solid phase to said complexes to form bound
and unbound complexes;

separating the bound and unbound complexes and
washing; and

10 measuring either the amount of bound complex or
unbound complex to quantify the amount of
vitamin B₁₂ present in the sample.

15 6. The process of claim 5 wherein the monoclonal
antibodies are labelled with a hapten molecule.

7. The process of claim 6 wherein said hapten molecule
is fluorescein isothiocyanate.

20 8. The process of claim 5 wherein said intrinsic factor
is labelled with an enzyme.

25 9. The process of claim 5 wherein in step (e) the
amount of intrinsic factor:antibody is measured, said
value being inversely proportional to the amount of
vitamin B₁₂ present in the sample.

30 10. The process of claim 5 wherein in step (e) the
amount of intrinsic factor:vitamin B₁₂ is measured, said
value being directly proportional to the amount of
vitamin B₁₂ present in the sample.

35 11. The process of claim 5 wherein step (c) involves
contacting the sample with antibodies specific to the
label used for the monoclonal antibodies of step (b),
coupled with magnetizable particles, to form a complex

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with the intrinsic factor:antibody complex, and the separation step (d) is by magnetic particle separation.

5 12. An immunoassay for determining the concentration of
vitamin B₁₂ in a sample comprising monoclonal antibodies
that bind to the intrinsic factor:vitamin B₁₂ complex said
monoclonal antibodies being labelled with a first label,
intrinsic factor labelled with a second label, and a
10 solid phase containing a capture ligand for one of said
labels with the other label being a signal generating
label for use in detection.

15 13. Monoclonal antibodies specific for the intrinsic
factor:vitamin B₁₂ complex.

14. The monoclonal antibodies of claim 13 which are
produced by the cell line 7E4.

20 15. A bound complex wherein intrinsic factor having a
first label is bound to vitamin B₁₂ forming intrinsic
factor:vitamin B₁₂ complex, which is bound to a monoclonal
antibody specific for the intrinsic factor:vitamin B₁₂
binding site having a second label, where one of said
labels is capable of being captured by a capture ligand
25 on a solid phase and the other label is a signal
generating label for use in detection.

30 16. A process for the determination of the concentration
of vitamin B₁₂ in a fluid containing vitamin B₁₂ bound to
binding proteins, comprising the steps of:

treating a sample of the fluid to free vitamin B₁₂
from the binding proteins;

35 contacting the sample with labelled intrinsic
factor, in excess, and labelled monoclonal
antibodies specific to the intrinsic

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factor: vitamin B₁₂ complex, in excess, to form
intrinsic factor: vitamin B₁₂ antibody complexes;

adding a solid phase to said complexes to form bound
and unbound complexes;

separating the bound and unbound complexes and
washing; and

measuring either the amount of bound complex or
unbound complex to quantify the amount of
vitamin B₁₂ present in the sample.

17. The process of claim 16 wherein said intrinsic
factor is labelled with a hapten molecule.

18. The process of claim 17 wherein said hapten molecule
is fluorescein isothiocyanate.

19. The process of claim 16 wherein the monoclonal
antibodies are labelled with an enzyme.

20. The process of claim 16 wherein in step (e), the
amount of intrinsic factor: vitamin B₁₂ is measured, said
value being directly proportional to the amount of
vitamin B₁₂ present in the sample.

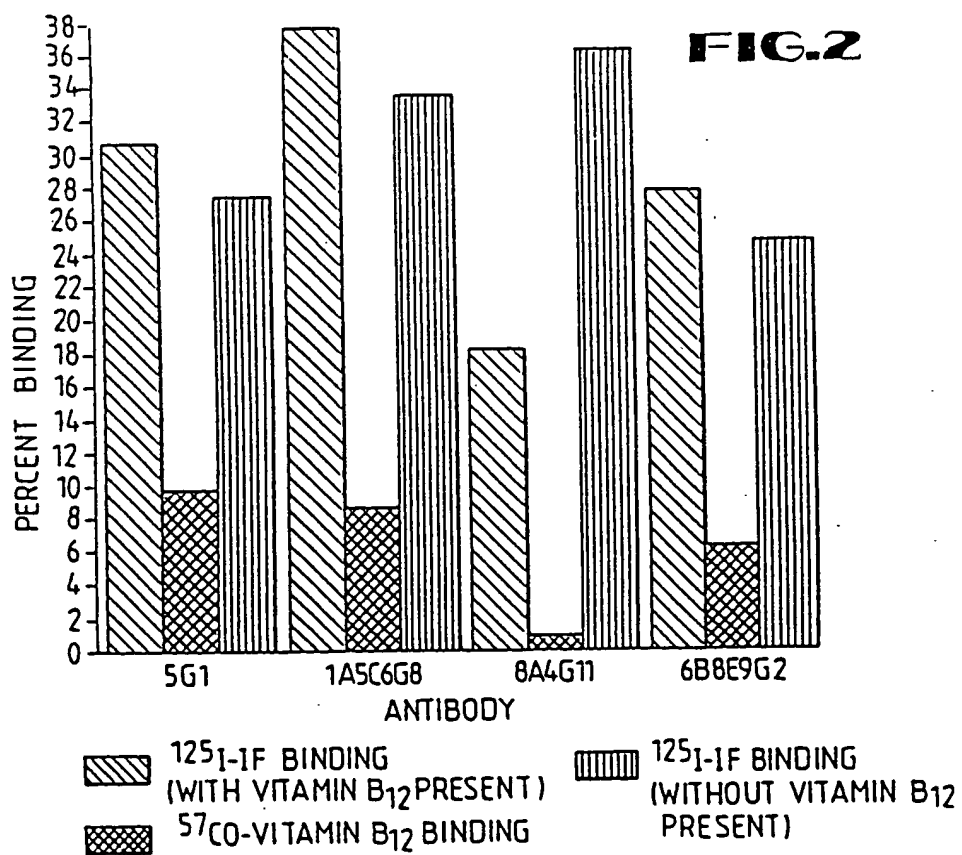
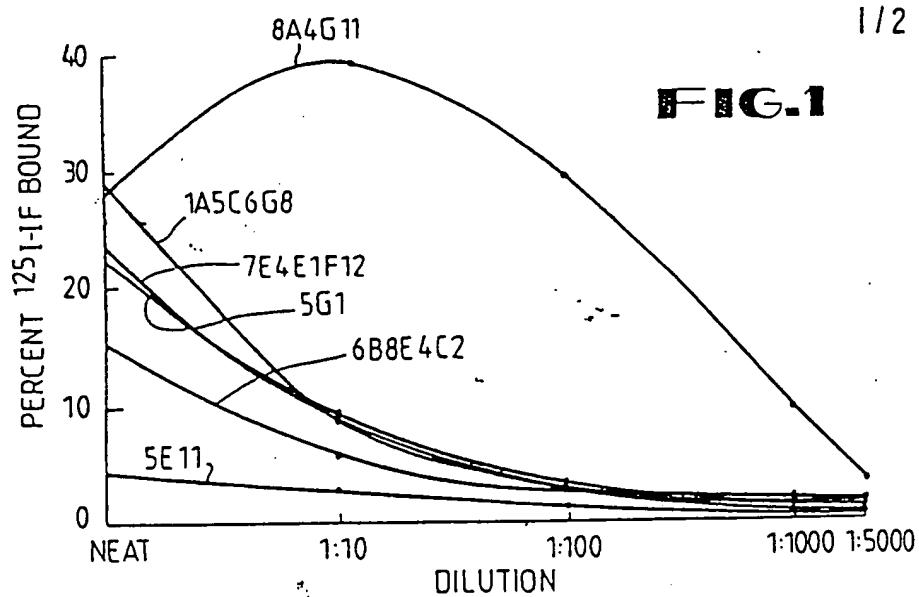
21. The process of claim 16 wherein in step (e), the
amount of excess monoclonal antibodies is measured, said
value being inversely proportional to the amount of
vitamin B₁₂ present in the sample.

22. The process of claim 16 wherein step (c) involves
contacting said sample with antibodies specific to the
label used for the monoclonal antibodies of step (b),
coupled with magnetizable particles, to form a complex
with the intrinsic factor: vitamin B₁₂: antibody complex,

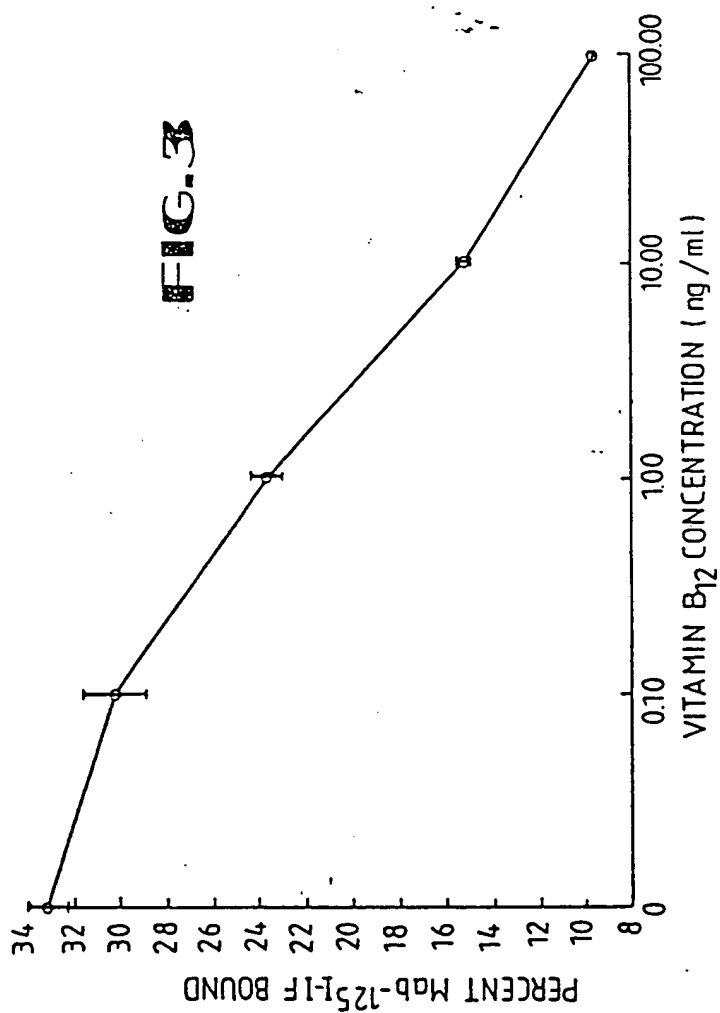
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and the separation step (d) is by magnetic particle separation.

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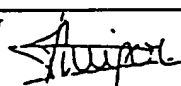


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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/03416

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC5: G 01 N 33/53, 33/577, 33/82, C 07 K 15/28		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	G 01 N; C 07 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	US, A, 4447528 (JAMES E. ELLIS ET AL.) 8 May 1984, see the whole document --	1-11
A	Chemical Abstracts, volume 69, no. 21, 18 November 1968, (Columbus, Ohio, US), Samloff I. et al.: "Rabbit blocking and binding antibodies to human intrinsic factor and intrinsic factor-B12 complex", see page 794, abstract 85058x, & J. Immunol. 1968, 101(3), 578- 586 --	2,3
A	US, A, 3981863 (GORDON DEAN NISWENDER ET AL.) 21 September 1976, see the whole document --	1,12
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
15th October 1990	29 OCT 1990	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	Mme N. KUIPER 	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
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Y	Chemical Abstracts, volume 82, no. 1, 6 January 1975, (Columbus, Ohio, US), Van de Wiel, D.F.M. et al.: "Production and purification of antibody against protein-vitamin B12 conjugates for radioimmunoassay purposes ", see page 167, abstract 1801s, & Clin. Chim. Acta 1974, 56(2), 143- 149 --	12-22
Y	EP, A2, 0238353 (SERONO DIAGNOSTICS PARTNERS (A MASSACHUSETTS LIMITED PARTNERSHIP)) 23 September 1987, see fig. 2, page 1 and page 4, lines 37-63 --	12-22
A	National Library of Medicinedatabase, Medline, File Med 77, NLM Accession no. 77258759, O'Brien HA et al: "A radioisotope dilution assay for unlabelled vitamin B12-intrinsic factor complex employing the binding intrinsic factor antibody; probable evidence for two types of binding antibody", & Scand J Haematol 1977 Aug; 19(2):201-6 --	12-22
A	Chemical Abstracts, volume 101, no. 7, 13 August 1984, (Columbus, Ohio, US), Shepherd H.A. et al.: "The preparation of human intrinsic factor-cobalamin complex from human gastric juice by immunoabsorption ", see page 280, abstract 50981v, & Clin. Chim. Acta 1984, 139(2), 155- 165 --	13
A	WO, A1, 8806632 (ADVANCED MAGNETICS, INC.) 7 September 1988, see pages 44-47 -- -----	22

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The European

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For more detail

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ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 90/03416

SA 38305

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 27/09/90
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For more details about this annex : see Official Journal of the European patent Office, No. 12/82